Biochemical Genetics, Vol. 35, Nos. 5/6, 1997

Proboscidean DNA from Museum and Fossil Specimens: An Assessment of Ancient DNA Extraction and Amplification Techniques

Hong Yang,[1,2] Edward M. Golenberg,[1] and Jeheskel Shoshani[1,3]

Received 10 Feb. 1997—Final 23 Apr. 1997

Applications of reliable DNA extraction and amplification techniques to postmortem samples are critical to ancient DNA research. Commonly used methods for isolating DNA from ancient material were tested and compared using both soft tissue and bones from fossil and contemporary museum proboscideans. DNAs isolated using three principal methods served as templates in subsequent PCR amplifications, and the PCR products were directly sequenced. Authentication of the ancient origin of obtained nucleotide sequences was established by demonstrating reproducibility under a blind testing system and by phylogenetic analysis. Our results indicate that ancient samples may respond differently to extraction buffers or purification procedures, and no single method was universally successful. A CTAB buffer method, modified from plant DNA extraction protocols, was found to have the highest success rate. Nested PCR was shown to be a reliable approach to amplify ancient DNA templates that failed in primary amplification.

KEY WORDS: ancient DNA; cetyltrimethylammonium bromide extraction; nested polymerase chain reaction; proboscideans.

INTRODUCTION

Ancient DNA retrieved from postmortem bones and soft tissues provides scientists with powerful molecular evidence to examine the genetic changes and phylogenetic relationships between extant and extinct taxa, even when morpho-

[1] Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202.
[2] To whom correspondence should be addressed at Department of Human Genetics, University of Michigan Medical School, Medical Science II M4708, Ann Arbor, Michigan 48109.
[3] Cranbrook Institute of Science, Bloomfield Hills, Michigan 48304.

0006-2928/97/0600-0165$12.50/0

logical and anatomical information is limited. Millions of museum specimens of both extant and extinct organisms that were collected from around the world at great expenses have become a new source of material for molecular investigations (Thomas, 1994). Using the polymerase chain reaction (PCR), DNA sequences have been obtained from both animal soft tissue and bone samples from old museum collections of contemporary species (Wayne and Jenks, 1991; Cooper *et al.,* 1992; Krajewski *et al.,* 1992) and from fossil material (Janczewski *et al.,* 1992; Hänni *et al.,* 1994; Höss *et al.,* 1996; Yang *et al.,* 1996). However, studies of highly degraded DNA are often confounded with problems that lead to a low efficiency of PCR amplification due to the presence of chemical inhibitors and, thus, hamper ancient DNA research from reaching its full potential in evolutionary and forensic studies.

Because only limited amounts of endogenous DNA survive postmortem degradation in old specimens, the efficiencies of DNA isolation and PCR amplification become critical for further DNA analysis. Different extraction techniques have been successfully applied to ancient animal specimens (for examples see papers in Herrmann and Hummel, 1994), and these methods can be grouped into two basic categories: the traditional proteinase K-based organic methods (Cooper, 1994; Hagelberg, 1994) and the glass bead approach (Höss and Pääbo, 1993; Cano and Poinar, 1993). Although these techniques have shown certain degrees of success on particular specimens, comparisons among these methods using ancient animal samples have rarely been made (for plant materials see Rogers, 1994). The low copy number of target DNA and the presence of inhibitors in ancient samples reduce the efficiency of PCR amplification. Several approaches including changes of $MgCl_2$ and bovine serum albumin (BSA) concentrations (Cooper, 1994), modified biphasic booster PCR (Hummel *et al.,* 1992), and nested PCR (Salo *et al.,* 1994) have been introduced to improve PCR efficiency in ancient DNA amplification. But due to the risk of contamination, the practical value of using two stage nested PCR has been uncertain.

The purpose of this paper is to test several commonly used ancient DNA extraction methods using tissue from museum and fossil proboscideans (elephants and their extinct relatives) of different ages and states of preservation. A CTAB-based isolation method that has shown high success rates on various ancient samples in our experiments is described. We also intend to show the effectiveness of using nested PCR techniques to improve PCR sensitivity during amplification for DNA extracted from ancient bone material. We demonstrate these techniques by using museum and fossil animal specimens that were preserved under dry conditions, but the results should have a general implication for other types of ancient materials including forensic samples. Extraction techniques dealing with sample preserved in liquid media are not included in this paper, and interested readers may consult Vachot and Monnerot (1996) for recent progresses.

MATERIALS AND METHODS

Proboscidean Samples and Laboratory Controls

Proboscidean specimens, ranging in age from 2 to over 46,000 years, were collected from Quaternary deposits and museums (Table I). Contemporary skin and muscle samples were obtained from three extant elephants: *Elephas maximus* (Asian elephant, EL#1), *Loxodonta africana* (African elephant, EL#4), and a putative intergeneric hybrid elephant (EL#3) between a male African elephant and a female Asian elephant. Air-dried skin from a frozen carcass of an extinct *Mammuthus primigenius* (woolly mammoth, EL#2) and bones from two extinct species, *Mammuthus primigenius* (woolly mammoth, EL#19) and *Mammut americanum* (American mastodon, EL#6, EL#23, EL#32, EL#29) were used. A blind testing system, aimed to check the accuracy of laboratory techniques and to avoid bias in sequence analysis, was designed as described by Yang *et al.* (1996, 1997).

The experiment was carried out in a plant molecular biology laboratory where no mammalian DNA (except for human DNA) was previously handled. Equipment and reagents designated solely for ancient DNA work were used. If reusable glassware was used, they were soaked in 0.5% sodium hypochlorite from 2 hr up to overnight and then were exposed to UV light in a UV cross-linker (Stratalinker Model 2400, Stratagene, CA) for 1 hr before use. Extraction buffers were also UV treated prior to DNA isolation. Extraction and PCR negative controls (reagents without tissue) were used in parallel with samples on all occasions. Preparation of PCR reagents was carried out in a laminar flow hood, and the PCR reaction solution was treated under UV light for 45 min before adding enzyme and DNA template. When two-stage nested PCR was performed, both extraction and primary PCR negative controls were carried along in the nested PCR as secondary controls. To test reproducibility, we sequenced PCR products amplified from genomic DNA that was obtained by different isolation techniques from the same specimen. For samples from which DNA was successfully isolated by only one method, at least two PCR amplifications obtained from independent extractions were sequenced.

DNA Extraction and a Modified CTAB Protocol

Samples were surface sterilized by washing them with 0.5% sodium hypochlorite and then rinsed with running deionized distilled water (ddH_2O) for 5 min. Between 0.15- and 0.5-g dust samples were collected by drilling into the material using a hand drill with a disposable 3-mm bit. Ethylenediaminetetraacetate (EDTA) treatment was then employed to decalcify bone samples. The dust samples were incubated with 10 vol (w/v) of 0.5 *M* EDTA at 37°C with constant

Table I. Proboscidean Samples and Extraction Methods Used in This Study

Taxon, sample number and name[a]	Nature of sample	Sample storage[b] and label number in the museum	Age (radiocarbon date in years before present) and origin of sample	Extraction methods tested[c]	Reference
EL#1. *Elephas maximus* (Iki)	Skin preserved in salt	WSUMNH	(14), died in 1980	a, B, C	Shoshani *et al.* (1982)
EL#2. *Mammuthus primigenius* (Lyakhovskiy mammoth)	Air-dried skin from frozen specimen	MNHN #HZ	(>46,000) Lyakhovskiy Island, Siberian Arctic, Russia	a, b, C	J. Shoshani (unpublished data)
EL#3. A hybrid between the two extant elephant genera[d] (Motty)	Skin preserved in unknown chemical preservatives	BMNH	(16), died in 1978	a, B, C	Lowenstein and Shoshani (1996)
EL#4. *Loxodonta africana* (Mtoto)	Skin preserved in salt	Brookfield Zoo, Brookfield, IL	(2), died in 1992	A, B, C	J. Shoshani (unpublished data)
EL#5. *Elephas maximus* (Iki)	Air dried muscle	WSUMNH	(14), died in 1980	a, B, C	Shoshani *et al.* (1982)
EL#6. *Mammut americanum* (Shelton)	Cranial fragment	WSUMNH	(12,320) Oakland County, MI	a, b, c	Shoshani *et al.* (1989)
EL#19. *Mammuthus primigenius* (Alaska mammoth)	Cranial fragment	AMNH #FAM A 2001-D	(20,000) Cripple Creek, AK	A, b, c	J. P. Alexander (pers. comm., 1994)
EL#23. *Mammut americanum* (Elmer)	Rib fragment	OCC	(10,200) Oakland County, MI	a, b, C	Shoshani *et al.* (1985)
EL#32. *Mammut americanum* (Elmer)	Rib fragment	OCC	(10,200) Oakland County, MI	a, b, C	Shoshani *et al.* (1985)
EL#29. *Mammut americanum* (not named)	Cranial fragment	WSUMNH	Late Pleistocene, radiocarbon date not available	a, b, c	J. Shoshani (unpublished data)

[a]The nickname or identification of the animal is given in parentheses.

[b]Museum abbreviations: WSUMNH, Wayne State University Museum of Natural History (Detroit, MI); MNHN, Museum National d'Histoire Naturelle (Paris, France); BMNH, British Museum of Natural History (London, England, New Name: Natural History Museum); AMNH, American Museum of Natural History (New York); OCC, Oakland Community College, Highland Lake campus (Union Lakes, MI).

[c]A or a, proteinase K organic extraction method (Cooper, 1994; Hagelberg, 1994); B or b, glass bead extraction method (Höss and Pääbo, 1993; Cano and Poinar, 1993); C or c, CTAB extraction method (Doyle and Doyle, 1987; Golenberg, 1994). Capital letter indicates success in obtaining amplifiable endogenous DNA, whereas small letter represents failure.

[d]The putative intergeneric hybrid between the two modern elephants, Loxodonta and Elephas, had an Asian elephant mother. The male elephant calf died shortly after his birth on July 11, 1978, at the Chester Zoo in England. The preserved specimen was prepared by a private company for the museum, and the chemical preservatives used to treat the specimen were not revealed.

slow shaking, and two to three changes of fresh EDTA solution were made during a 2-day period until the solution became colorless. Samples were divided equally for the following three DNA extraction approaches: (a) proteinase K method (Cooper, 1994; Hagelberg, 1994), (b) glass bead approach (Höss and Pääbo, 1993; Cano and Poinar, 1993), and (c) 2% CTAB-based method modified from isolation protocols for plant tissues (Doyle and Doyle, 1987; Golenberg, 1994) (see below for a detailed protocol) (Table I). Two protocols involving different proteinase K extraction buffers were used (Cooper, 1994; Hagelberg, 1994). For the glass bead approach, the glassmilk–NaI method (Cano and Poinar, 1993) was applied for both soft tissue and bone specimens, and the silica–GuSCN (guanidinium thiocyanate) method (Höss and Pääbo, 1993) was applied for all bone samples.

The third extraction approach, involving 2% (w/v) cetyltrimethylammonium bromide (CTAB) buffer, is briefly described here. About 0.15 g of soft tissue or bone dust was placed in a presterilized mortar, and 600 µl of preheated (65°C) extraction buffer containing 2% CTAB, 100 m*M* Tris–HCl (pH 8.0), 1.4 *M* NaCl, 20 m*M* EDTA, and 0.2% 2-mercaptoethanol (w/v) was added in a stepwise manner (200 µl each time, for three times) during grinding. The ground sample with buffer was transferred into a 1.5-ml tube, and after vigorous vortexing for 2 min, the tube was incubated at 65°C for 30 min to 1 hr. The supernatant was collected after centrifugation at 13,000 rpm for 20 min, and an equal volume of chloroform:isoamyl alcohol (24:1) was added. After 2 min of centrifugation, the upper aqueous phase was transferred into a new tube, and 1.5 vol of 75% cold ethanol and 50 µl (0.1 vol) of 3 *M* NaOAc were added for overnight precipitation at −20°C. The pellet was collected by centrifugation for 20 min at 13,000 rpm and was washed twice with 75% ethanol. Finally, the pellet was vacuum-dried and then reconstituted in 50 µl 1× TE.

In addition to a separate silica–GuSCN extraction (Höss and Pääbo, 1993), cross-testing between different lysis buffers (2% CTAB, proteinase K with *N*-lauroylsarcosine, and proteinase K with SDS and DTT) and purification procedures (glassmilk, chloroform, and phenol/chloroform) was performed on the bone samples (Fig. 1). For each sample (usually 1 g bone dust), the aqueous layer was collected as a stock after 18 h of incubation at 55°C with one of the three tested buffers. The stock was then divided equally (usually 700 µl each) for further extraction using one of the purification procedures (Fig. 1). A 5-µl aliquot of the extraction result was examined in a 0.7% agarose minigel containing 0.6% chemically modified galactomannan (Synergel, Diversified Biotech, MA) with ethidium bromide staining. Because previous investigators have encountered problems when using UV absorption to estimate DNA concentration from ancient soft tissue (Pääbo, 1989) or bone material (Tuross, 1994), spectrophotometry was not performed on these extracts.

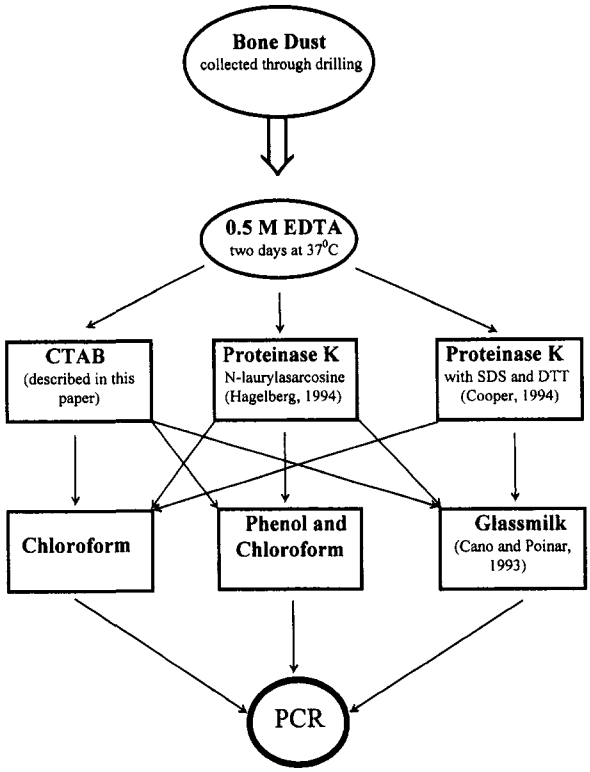

Fig. 1. Flowchart showing cross assessment between different extraction buffers and purification techniques for DNA extraction from ancient bones.

PCR Amplification and Sequencing

PCR amplification, targeting various lengths of the cytochrome *b* gene of the mitochondrial genome (mtDNA), was attempted on all extractions. Primers flanking different lengths of the cytochrome *b* gene were published previously (Irwin *et al.*, 1991; Yang *et al.*, 1996, 1997). The PCR reaction solution (for 25-μl reactions) consisted of 2.5 μl of 10× PCR buffer (500 m*M* KCl, 100 m*M* Tris–HCl, and 1% Triton X-100), 2.5 μl of each primer (5 μ*M*), 2.5 μl of each dGTP, dATP, dTTP, and dCTP (2 m*M* each), 4 μl of $MgCl_2$ (25 m*M*), and 8.375 μl of ddH_2O. The reaction mixture was exposed to UV light for 45 min to cross-link double-stranded DNA before adding 2.5 μl of DNA template and enzyme, thereby reducing the possibility of contamination. The PCR cocktail was covered by 2 drops (about 40 μl) of mineral oil and was run on a heat-block based thermal cycler (Coy Model 110S) with the following program: 3 min of initial denatur-

ation at 94°C followed by 40 cycles consisting of denaturation at 94°C (40 sec), annealing at 50°C (40 sec), and extension at 72°C (1 min). The *Taq* DNA polymerase (0.125 μl, 5 U/μl) was added after the 3-min initial denaturation. When the PCR product from the primary amplification was too weak for further sequencing, and optimization of PCR conditions could not improve the efficiency, two-stage nested PCR amplification was performed. In the nested PCR, the condition for the primary amplification was the same as described above, and the primary PCR product was directly used for secondary amplifications without further purification. A pair of internal primers [Elcytb65 and Elcytb320R (Yang *et al.*, 1997)] was used for another 40 cycles of amplification on a capillary-based air thermal cycler (ATC Model 1605, Idaho Technology, ID). The reaction buffer (5×) for the secondary amplification was different from that of the primary amplification and consisted of 250 m*M* Tris (pH 8.5), 5 m*M* $MgCl_2$, 100 m*M* KCl, and 2.5 mg/ml BSA. For a 50-μl reaction, the buffer is mixed with 5 μl of each primer (5 μ*M*), 5 μl of each dNTPs (2 m*M* each), 4 μl $MgCl_2$ (25 m*M*), 0.25 μl of Taq DNA polymerase (5 U/μl), 15.75 μl of ddH_2O, and 5 μl of template DNA from the primary reaction. The reaction solution was loaded in a capillary tube provided by the manufacturer, and both ends of the capillary tube were sealed by flame. The capillary tube was frozen at −20°C for 5 min and was then inserted into the instrument. After the reaction chamber reached 80°C, the reaction tubes were put into the thermal cycler. The PCR thermal profile for the secondary amplification consisted of denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C (12 sec), annealing at 50°C (12 sec), and extension at 72°C (30 sec). The PCR product was examined using agarose gel electrophoresis.

We established the identity of the origins of the PCR products by direct sequencing of the double-stranded PCR product following the protocols of Barnard *et al.* (1994) and Yang *et al.* (1996). Sequences obtained in the laboratory were aligned with the previously published *Loxodonta* sequence (Irwin *et al.*, 1991) using the computer software Sequencher 2.1 (Gene Codes Co., MI) and analyzed using both maximum parsimony (Swofford, 1991) and distance (Felsenstein, 1990) approaches.

RESULTS

DNA Extraction

After ethidium bromide staining, DNA up to 10 kilobases (kb) was visible under UV light from extractions of the 2-year-old *Loxodonta* (EL#4) obtained using all three tested methods [CTAB method (Cooper, 1994; Cano and Poinar, 1993)]. In contrast, low molecular weight DNA from the 14-year-old *Elephas* (EL#1 and EL#5), the 46,000-year-old *Mammuthus* (EL#2), and the 16-year-old hybrid (EL#3), was visible only in CTAB-based extractions (Fig. 2). The majority of the

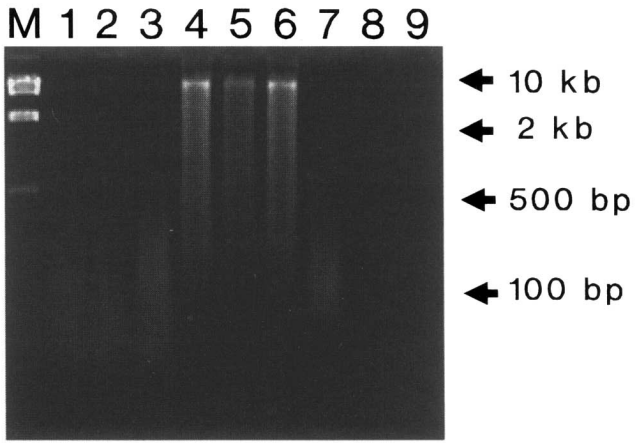

Fig. 2. Agarose gel electrophoresis of extracted DNA stained with ethidium bromide, showing the size distribution of genomic DNA from proboscidean samples. A 5-μl aliquot of 50 μl extraction stock was loaded in each well. Lane M, molecular size marker (λ DNA cut by *Hin*dIII, 0.25 μg/μl); lane 1, EL#1 (CTAB method); lane 2, EL#2 (CTAB method); lane 3, EL#3 (CTAB method); lane 4, EL#4 (proteinase K/phenol method); lane 5, EL#4 (glass-milk method); lane 6, EL#4 (CTAB method); lane 7, EL#5 (CTAB method); lane 8, El#19 (proteinase K/phenol method); lane 9, EL#23 (CTAB method).

low molecular weight DNA was below 500 base pairs (bp). Small quantities of endogenous DNA from EL#1, EL#3, and EL#5 were obtained using the glassmilk–NaI method as later substantiated by PCR amplification and sequencing but were too low to be visualized by ethidium bromide staining. The proteinase K/phenol approach failed to yield amplifiable DNA from all soft tissue samples except EL#4. No visible DNA was extracted from the tested bone material, but amplifiable DNA was obtained from three bone samples, the 20,000-year-old *Mammuthus* (EL#19) and the 10,200-year-old *Mammut* (EL#23 and EL#32). When the combination between extraction buffer from Cooper (1994) and the organic purification procedure of Hagelberg (1994) was used, extraction from EL#19 yielded amplifiable DNA. We also obtained amplifiable DNA from EL#23 and EL#32 using the CTAB method. The ancient origin of the DNA amplified from bone extractions was later supported by sequencing analysis. We did not obtain amplifiable DNA using the method described by Höss and Pääbo (1993) from the tested bone material. Two other Pleistocene mastodon bones (EL#6 and EL#29) collected from other Michigan sites did not yield amplifiable DNA using any of the above extraction methods (Table I).

PCR Amplification

A 228-bp segment of the cytochrome *b* gene could be amplified from DNA extractions that were visible under ethidium bromide staining with a single run of

40 cycle PCR with 4 m*M* $MgCl_2$ concentration. The entire cytochrome *b* gene (about 1.2 Kb) could be amplified using primers T1 and B2 of Irwin *et al.* (1991) from the 2-year-old *Loxodonta* (EL#4). An inverse relationship between amplification efficiency and amplification length was observed when amplifying 228 bp, 459 bp, and 1.2-kb fragments of the sample EL#4 (data not shown). Using CTAB extracted DNA as templates, fragments up to 459 bp were obtained from samples of the *Elephas* (EL#1 and EL#5), and the hybrid (EL#3) in a single run of 40 cycles, although different amplification efficiency was clearly noticed for each CTAB extracted DNA template under the same PCR condition (Fig. 3). Only a weak band was detected from the *Mammut* sample EL#23 obtained from the CTAB method during a single run of amplification (40 cycles) targeting a small fragment (228 bp). The low efficiency could not be improved by varying $MgCl_2$ and/or BSA concentrations, using alternative primers, or changing annealing temperatures. This low efficiency could be circumvented, however, by employing nested PCR. When a pair of internal primers was used in the secondary PCR, high-quality amplification product was obtained (Fig. 4). Similarly, nested PCR also yielded amplifications from a *Mammuthus* (EL#19) and the *Mammut* (EL#32) from which primary PCR failed to produce a product. No fragment longer than 228 bp was amplified from the bone samples even when the nested PCR technique was used.

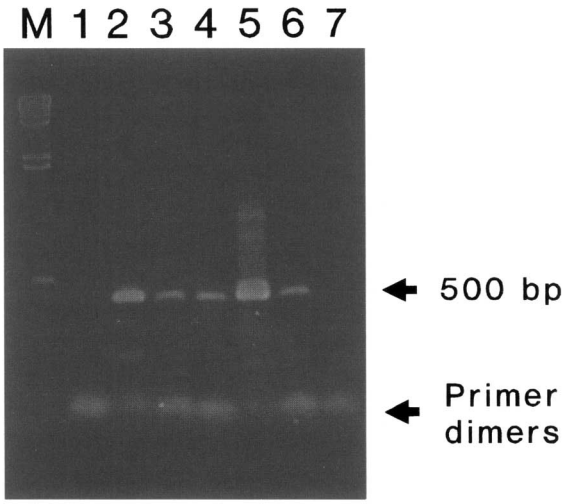

Fig. 3. Result of PCR amplifications of 459-bp fragments showing different PCR efficiencies for different templates under the same amplification condition. DNA templates for the amplifications were extracted using the CTAB method described in this paper. A 5-μl aliquot was loaded to each well. Lane M, molecular size marker (λDNA cut by *Hin*dIII, 0.25 μg/μl); lane 1, PCR negative control; lanes 2–6, EL#1–EL#5; lane 7, extraction control.

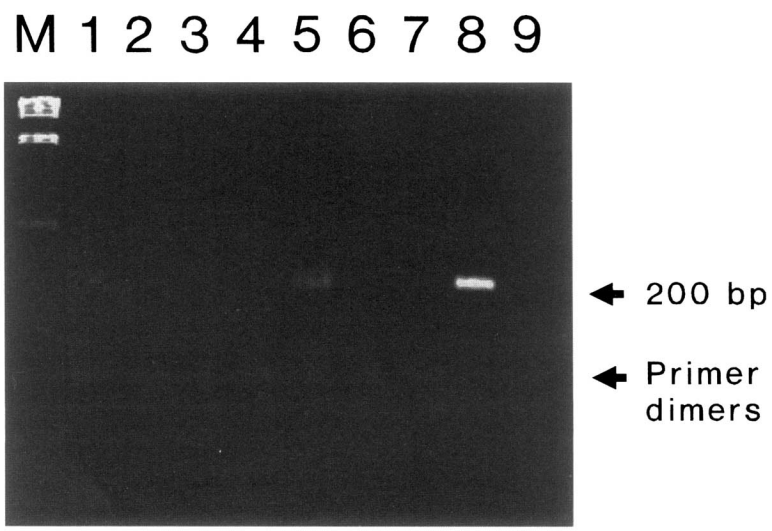

Fig. 4. Result of PCR amplifications for the bone sample EL#23 extracted using the CTAB method, showing the contrast of PCR efficiency between primary and nested PCR amplifications. Lane M, molecular size marker (λDNA cut by *Hin*dIII, 0.25 μg/μl); lane 1, PCR negative control; lane 2, EL#23 sample; lane 3, extraction control (primers T1 and B2 were used in lanes 1–3 for a single run of 40 cycle); lane 4, PCR negative control; lane 5, EL#23 sample; lane 6, extraction control (primers Elcytb65 and Elcytb320R were used in lanes 4–6 for a single run of 40 cycles); lane 7, PCR negative control carried from the primary amplification; lane 8, EL#23 nested PCR amplification; lane 9, extraction control carried from primary amplification. During the nested PCR (lanes 7–9), primers T1 and B2 were used for 40 cycles of primary amplification, and then primers Elcytb65 and Elcytb320R were used for the nested PCR using 5 μl primary PCR product as template. A 5-μl aliquot was loaded in each well.

Authentication of Ancient DNA Sequences

Authentication of the endogenous origin of the amplified DNA was established through nucleotide sequence analysis by following a blind testing system (Yang *et al.,* 1997). The cytochrome *b* sequence fragments were deposited in the GenBank with the following accession numbers: *E. maximus* U23740, *L. africana* U23741, *M. primigenius* U23738 and U23739, and *M. americanum* U23737. Completely aligned sequences from studied samples and a detailed phylogenetic analysis were published elsewhere (Yang *et al.,* 1996). While the 228-bp sequence from different specimens vary in 1 to 13 nucleotides, identical sequences were obtained from different samples collected from the same individual animal under a blind testing design. Reproducible sequences were also obtained from the same samples extracted using different isolation methods and PCR amplified at different occasions. Phylogenetic analysis of these sequences (except for the hybrid, which differs from the *Elephas* only by one nucleotide) has shown that all

proboscidean taxa clustered together to form a monophyletic clade with the *Mammut* sequence branching out first. Such a phylogenetic reconstruction matches a proboscidean systematics based on morphological (Shoshani, 1986; Tassy, 1996) and previous immunological (Shoshani *et al.,* 1985) results—supporting the authenticity of the ancient DNA sequences (Yang *et al.,* 1996).

DISCUSSION

The major challenges in ancient DNA extraction are to maximize the DNA yield, to eliminate inhibitors that block PCR amplification, and to minimize the possibility for outside contamination. The CTAB-based extraction method employed in this study consistently yielded visible DNA from old soft tissue, and the extracted DNA could be readily amplified. Previous work has shown that low molecular weight DNA is commonly obtained from ancient human and animal soft tissue (Doran *et al.,* 1986; Pääbo *et al.,* 1988, 1989; Gaensslen and Berka, 1994), although the visible nucleic acid may be a mixture of both endogenous (the sample) and exogenous (e.g., bacteria) origins. In our proboscidean samples, the size distribution of isolated DNA from soft tissue generally correlates with the length that can be amplified by PCR, largely reflecting the quantity and quality of DNA from the sample itself. We noticed that the glass bead approach constantly gave less DNA when compared with the other two methods. For example, DNA extracted using the glassmilk–NaI method from the 2-year-old *Loxodonta* (EL#4) yielded about half the amount obtained with the other two methods, and no visible DNA was obtained using the glassmilk–NaI method from other soft tissue samples from which the CTAB-based method provided low molecular weight DNA (Fig. 2). Our result is also consistent with previous observations that endogenous DNA can be amplified from bone extractions that did not give visualization of genomic DNA (Hänni *et al.,* 1994). Whereas high success rates in DNA retrieval from ancient material using the silica–GuSCN method were reported (Höss and Pääbo, 1993; Handt *et al.,* 1994), this method did not yield amplifiable DNA from the tested bone material in our study. Of course, this negative result may reflect our inability to obtain amplifiable DNA from the limited bone samples in this study. As suggested by Hänni *et al.* (1994), the failure could be due to the small amount of sample (0.15 g) used. It has been noted that genomic DNA obtained using the traditional proteinase K buffer followed by phenol and chloroform/isoamyl alcohol purification procedure may contain known (e.g., fulvic acid) and unknown inhibitors or result in DNA loss during the isolation process (Tuross, 1994; Cattano *et al.,* 1995). In our experiments, except for EL#4, no amplifiable DNA was extracted from tested soft tissue using the phenol/chloroform based purification method. However, the bone sample EL#19 yielded endogenous DNA using the buffer described by Cooper (1994) followed by phenol purification method of Hagelberg (1994). These results indicate that

although the CTAB-based method has a better success rate, especially on soft tissue, no current extraction technique is universally applicable to ancient animal material. In addition, our findings may also suggest that the choice of extraction buffer may be critical for the success of ancient DNA isolation. When using the buffer described by Hagelberg (1994), a white coprecipitate occurred in all soft tissue extractions during ethanol or isopropanol precipitation, and this precipitation later inhibited *Taq* polymerase. But a simple substitution, using the buffer described in Cooper (1994), eliminated this problem. Because very limited amounts of ancient nucleic acids are preserved in old specimens, it is critical to release as many molecules as possible during the initial isolation process, and a suitable lysis buffer certainly plays an important role in achieving this goal. It is reasonable to assume that due to the unique decay process of each specimen, chemicals presented in each sample as decay products may interfere with a given extraction process but respond favorably to another.

An efficient amplification producing enough DNA copies for subsequent direct sequencing is known to be dependent upon both the quality and quantity of DNA templates (Pääbo *et al.,* 1989). Among the tested samples, EL#4 not only provided high molecular weight DNA during extractions but also yielded longer PCR fragments indicating the high quality and large quantity of endogenous DNA preservation in this sample. In contrast, a relatively low efficiency was observed when older samples were used as template (Fig. 3), and no PCR product or only very weak amplification was detected when bone extractions were used. It has been suggested that low PCR efficiency may be caused by partial inhibition to *Taq* polymerase (Cooper, 1994; Tuross, 1994), but the effort using different BSA concentrations did not eliminate the problem. This low efficiency can be overcome by using two-stage nested PCR. When internal primers were used, strong amplifications were obtained, and the PCR product was proven to be endogenous by subsequent sequence analysis. Our applications of the nested PCR are consistent with previous reports of the high performance of the technique. Zimmermann *et al.* (1994) demonstrated the sensitivity and specificity of nested PCR in amplifications of target DNA in the presence of the vast majority of nontarget background DNA. Salo *et al.* (1994) successfully applied nested PCR technique to amplify ancient DNA that failed in single runs of primary amplification. It is apparent that during the secondary amplification, the priming efficiency between template DNA and internal primers is dramatically increased and nonspecific priming is largely reduced. The primers Elcytb65 and Elcytb320R were designed to include 3′ regions which differ between proboscideans and humans (Yang *et al.,* 1996; 1997). Thus, we expected that any contaminating human DNA, if present, would not be primed. Indeed, when we tested the primers using human DNA as template for a single run of 40 cycles, the expected band was not observed. We did not encounter human DNA contamination in sequences that were derived from primary amplifications. In several cases during the

secondary nested PCR, however, human sequences were obtained. It should be warned that the dramatic increase in sensitivity may be a risk for contamination, therefore, it is critical to include the primary negative controls as templates during the secondary amplification. The reason why nested PCR increases PCR efficiency is largely unknown, but recent experiments indicated that during PCR reactions, target and nontarget fragmented DNA templates are competing for dNTPs or enzymes. The fragmented DNA molecules take up dNTPs when overlapping fragments anneal and are extended by *Taq* polymerase mediated DNA polymerization. After nontargeted genomic DNA extension is exhausted largely during the primary amplification, the high molar concentration of reagents in the secondary PCR allows noncompetitive target PCR to produce large amounts of the desired DNAs (Golenberg *et al.,* 1996). Meanwhile, if inhibitors are present, they will be further diluted in the second round of amplification, allowing a more efficient PCR.

CONCLUDING REMARKS

Studies of ancient DNA from museum and fossil samples can provide valuable information toward a better understanding of degraded DNA preserved in postmortem specimens. This information helps to improve molecular techniques designed to recover and analyze old DNA to be used for evolutionary studies and as well as for forensic analysis. Our comparison of commonly used ancient DNA extraction techniques based on old proboscidean specimens indicates that, although none of the current extraction methods can be applied to ancient specimens with universal success, a CTAB-buffer based approach described in this paper consistently outperforms other methods. In contrast, glass bead-based methods usually cause noticeable loss of genomic DNA during purification. We also found that the choice of extraction buffer may be critical to the success of recovering endogenous DNA from different types of tissue (for example, soft tissue vs bone material) preserved under different physical and chemical conditions. We believe that modified two stage-nested PCR technique, which is capable of increasing amplification efficiency in the second amplification, is especially useful for study of decayed DNA that often produces negative result or only generates very weak products during first runs of amplification.

ACKNOWLEDGMENTS

We thank Ashley Byun (University of Victoria) for sharing her CTAB extraction information with us and John P. Alexander (American Museum of National History) for providing information regarding a mammoth sample (EL#19) that was used in the study. We are also grateful to Richard E. Tashian (University of Michigan) for reading the manuscript and for providing valuable suggestions.

This work was supported in part by an Alfred P. Sloan Postdoctoral Fellowship (93-4-6-ME) awarded to Hong Yang.

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